

University of Dundee

Assembly and structure of Lys33-linked polyubiquitin reveals distinct conformations

Kristariyanto, Yosua Adi; Choi, Soo-Youn; Rehman, Syed Arif Abdul; Ritorto, Maria Stella; Campbell, David G.; Morrice, Nicholas A.

Published in:
Biochemical Journal

DOI:
[10.1042/BJ20141502](https://doi.org/10.1042/BJ20141502)

Publication date:
2015

Licence:
CC BY

Document Version
Publisher's PDF, also known as Version of record

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Kristariyanto, Y. A., Choi, S.-Y., Rehman, S. A. A., Ritorto, M. S., Campbell, D. G., Morrice, N. A., Toth, R., & Kulathu, Y. (2015). Assembly and structure of Lys³³-linked polyubiquitin reveals distinct conformations. *Biochemical Journal*, 467(2), 345-352. <https://doi.org/10.1042/BJ20141502>

General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

ACCELERATED PUBLICATION

Assembly and structure of Lys³³-linked polyubiquitin reveals distinct conformations

Yosua Adi Kristariyanto*, Soo-Youn Choi*, Syed Arif Abdul Rehman*, Maria Stella Ritorto*, David G Campbell*, Nicholas A Morrice*¹, Rachel Toth* and Yogesh Kulathu*²

*MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, U.K.

Ubiquitylation regulates a multitude of biological processes and this versatility stems from the ability of ubiquitin (Ub) to form topologically different polymers of eight different linkage types. Whereas some linkages have been studied in detail, other linkage types including Lys³³-linked polyUb are poorly understood. In the present study, we identify an enzymatic system for the large-scale assembly of Lys³³ chains by combining the HECT (homologous to the E6–AP C-terminus) E3 ligase AREL1 (apoptosis-resistant E3 Ub protein ligase 1) with linkage selective deubiquitinases (DUBs). Moreover, this first characterization of the chain selectivity of AREL1 indicates its preference for assembling Lys³³- and Lys¹¹-linked Ub chains. Intriguingly, the crystal structure of Lys³³-linked diUb reveals that it adopts a

compact conformation very similar to that observed for Lys¹¹-linked diUb. In contrast, crystallographic analysis of Lys³³-linked triUb reveals a more extended conformation. These two distinct conformational states of Lys³³-linked polyUb may be selectively recognized by Ub-binding domains (UBD) and enzymes of the Ub system. Importantly, our work provides a method to assemble Lys³³-linked polyUb that will allow further characterization of this atypical chain type.

Key words: deubiquitinase, homologous to the E6–AP C-terminus (HECT) E3 ligase, polyubiquitin, ubiquitin linkage.

INTRODUCTION

Ubiquitylation is a reversible post-translational modification (PTM) that regulates many cellular processes, including protein degradation, endocytosis, DNA repair and immune response [1]. Addition of ubiquitin (Ub) to a substrate lysine involves a three-step enzymatic cascade involving Ub-activating enzyme (E1), Ub-conjugating enzymes (E2) and Ub-ligating enzymes (E3) [2]. E3 ligases fall into three main classes depending on their mechanism of Ub transfer to substrate. RING (really interesting new gene) ligases transfer Ub directly from the E2 on to substrate whereas HECT (homologous to the E6–AP C-terminus) ligases form a thioester intermediate with Ub before transfer on to substrate lysine [3,4]. RBR (RING-between-RING) ligases form the third class and employ a hybrid mechanism for catalysing Ub transfer [5].

PolyUb chains of eight different linkage types can be formed since the seven lysine residues (Lys⁶, Lys¹¹, Lys²⁷, Lys²⁹, Lys³³, Lys⁴⁸ and Lys⁶³) and N-terminal Met¹ residue in Ub can themselves accept another molecule of Ub [6]. PolyUb of some linkage types have been structurally characterized and these reveal distinct conformations for different linkages [7]. Crystal structures and solution studies using NMR reveal closed conformations for Lys⁶-, Lys¹¹- and Lys⁴⁸-linked diUb as a result of extensive interactions between the Ub moieties [8–12]. In contrast, Lys⁶³- and Met¹-linked diUb adopt extended conformations that lack intermoiety interactions [13]. Interestingly, alternate

conformations have been observed for some linkage types, highlighting the flexible nature of polyUb [14,15].

The topologically distinct polyUb linkages are recognized by Ub-binding domain (UBD)-containing proteins to couple ubiquitylation to diverse cellular responses [16]. For instance Lys⁴⁸-linked polyUb target proteins for proteasomal degradation, whereas Lys⁶³- and Met¹-linked polyUb chains have non-degradative roles in DNA damage response and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B-cells) signalling [1,17]. For the remaining linkage types, little is known about their precise cellular function.

Lys³³ chains may not be linked to proteasomal degradation, as the amounts of Lys³³ linkages do not increase following proteasome inhibition [18]. Further, several studies show that Lys³³ chains have non-degradative functions. T-cell antigen receptor (TCR) activation is negatively regulated in a proteolysis independent manner by Lys³³-linked polyUb, when the RING and HECT E3 ligases, Cbl-b (Casitas B-lineage lymphoma b) and Itch respectively modify the zeta-subunit of the TCR with this Ub chain type [19]. Lys³³-linkages are also reported to negatively regulate activity of AMPK (AMP-activated protein kinase)-related protein kinases in a non-degradative manner [20]. This linkage type has recently been linked to protein anterograde transport from the *trans*-Golgi network (TGN), where Lys³³-linked polyubiquitylation of coronin-7 (Crn7), an F-actin regulator, facilitates its targeting to the TGN, which promotes F-actin assembly at TGN and contributes to post-Golgi trafficking

Abbreviations: AREL1, apoptosis-resistant E3 ubiquitin protein ligase 1; ASU, asymmetric unit; Crn7, coronin-7; DUB, deubiquitinase; HECT, homologous to the E6–AP C-terminus; pRM, parallel reaction monitoring; PTM, post-translational modification; RBR, RING-between-RING; RING, really interesting new gene; TCR, T-cell antigen receptor; Ub, ubiquitin; UBD, ubiquitin-binding domain.

¹ Present address: AB Sciex UK Limited, Warrington, Cheshire WA1 1RX, U.K.

² To whom correspondence should be addressed (email ykulathu@dundee.ac.uk).

[21]. Interestingly, Lys³³-ubiquitylated Crn7 is recognized by the UBDs of the clathrin adaptor protein Epsin15 to result in translocation of Crn7 to the TGN.

Being a reversible PTM, ubiquitylation is regulated by deubiquitinases (DUBs) that hydrolyse isopeptide bonds between two Ub moieties or between Ub and the targeted protein [6,22]. The DUB TRABID (TRAF-binding domain-containing protein) was recently identified to preferentially hydrolyse Lys²⁹- and Lys³³-linkages [8,23,24]. Whereas TRABID was shown to regulate Wnt signalling, it is not clear if Lys³³-linked polyubiquitylation is involved [25]. Whereas these studies point to non-proteolytic roles for Lys³³ linkages in several cellular processes, we have a poor understanding of the ligases that can assemble Lys³³ linkages, the specific signals in response to which they are made, how they are decoded and disassembled.

In the present study, we focused on identifying ligases capable of assembling Lys³³ chains for biochemical and structural characterization. By screening a panel of HECT E3 ligases we identified the uncharacterized ligase AREL1 (apoptosis-resistant E3 Ub protein ligase 1; also known as KIAA0317) to assemble Lys³³ linkages along with other linkages. We then used linkage-selective DUBs to remove these additional linkages assembled by AREL1 to obtain pure Lys³³ chains. The enzymatic system we established allowed us to assemble large quantities of Lys³³-linked polyUb, thus enabling structural analysis of this atypical chain. We report the first crystal structures of Lys³³-linked diUb and triUb, which reveal distinct conformations.

MATERIALS AND METHODS

cDNA and antibody

All cDNA constructs used in the present study were generated by the DNA cloning team, Division of Signal Transduction Therapy, Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, University of Dundee United Kingdom (Supplementary Table S1). Recombinant proteins and plasmids generated for the present study are available from our reagents website (<https://mrppureagents.dundee.ac.uk/>). Anti-Ub antibody was purchased from SIGMA (U5379).

Protein expression and purification

Recombinant GST-fusion proteins were expressed in BL21 *Escherichia coli* cells. Cultures were grown in 2xTY media to D_{600} of 0.6–0.8 and the protein expression was induced by adding 300 μ M IPTG and further incubation at 16°C overnight. Cells were lysed by sonication in lysis buffer [50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 10 % glycerol, 0.075 % 2-mercaptoethanol, 1 mM benzamide, 1 mM PMSF and complete protease inhibitor cocktail (Roche)]. Bacterial lysate was clarified by centrifugation at 30 000 g for 30 min and incubated subsequently with Glutathione Sepharose 4B resin (GE Healthcare) for 2 h at 4°C. Resins were washed with high salt buffer (250 mM Tris, pH 7.5, 500 mM NaCl and 5 mM DTT) and low salt buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 10 % glycerol and 1 mM DTT). Purified proteins were eluted in low salt buffer supplemented by 30 mM glutathione or cleaved off from the GST-tag by incubating the beads with C3 protease overnight at 4°C.

Ubiquitylation assays

Analytical assays were carried out in 20 μ l of reaction mixtures containing 250 nM UBE1 (Ub-activating enzyme E1), 2.25 μ M

UBE2 (Ub-conjugating enzyme E2) (UBE2D1, D2, D3 or L3), 1.56 μ M HECT E3 (ITCH, AREL1, SMURF1 (SMAD ubiquitylation regulatory factor 1), SMURF2, UBE3C, HECW1 (HECT, C2 and WW domain-containing protein 1), HUWE1 (HECT, UBA and WWE domain-containing protein 1), WWP1 (WW domain-containing protein 1) or WWP2), 57 μ M Ub, 10 mM ATP, 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂ and 0.6 mM DTT for 3 h at 30°C. The reaction was quenched by addition of LDS (lithium dodecyl sulphate) sample buffer (Life Technology), resolved by SDS/PAGE on 4 %–12 % gradient gels and subjected to Western blot analysis using anti-Ub antibody. Where indicated, after 3 h reaction, a final concentration of 20 μ M Cezanne EK, 5 μ M OTUB1 (OTU domain-containing ubiquitin aldehyde-binding protein 1), 5 μ M TRABID and 5 mM DTT was added to the ubiquitylation reaction and incubated further for 2 h at 30°C.

Lys³³-linked polyUb assembly and purification

Large-scale Lys³³-linked polyUb chains assembly was carried out in 1.5 ml of reaction volume with 25 mg of Ub (Sigma), 500 nM UBE1, 9 μ M UBE2D1, 6.3 μ M AREL1, 10 mM ATP, 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂ and 0.6 mM DTT at 30°C for 6 h. To remove contaminating linkages, 20 μ M Cezanne E²⁸⁷K/E²⁸⁸K (Cezanne EK), 5 μ M OTUB1 and 5 mM DTT were added in to the assembly reaction and incubated further at 30°C overnight. The reaction mixture was diluted to a total volume of 50 ml of 50 mM sodium acetate (pH 4.5). Lys³³ chains of defined lengths were purified by cation exchange using a Resource S 6 ml column (GE Healthcare), equilibrated in buffer A (50 mM sodium acetate, pH 4.5) and eluted in a gradient with buffer B (50 mM sodium acetate, pH 4.5, 1 M NaCl).

Parallel reaction monitoring MS analysis

PolyUb chains were digested with trypsin and analysed on an LTQ-Velos mass spectrometer (Thermo) fitted with an Easy-Spray Source (Thermo) and utilizing a Dionex RSLC HPLC system. Standard diUb chains were purchased from Boston Biochemicals and a synthetic peptide AK(GG)IQDK representing the tryptic Ub K29 linkage was purchased from Pepceuticals. Digests (prepared in 0.1 % TFA (trifluoroacetic acid)/water) were concentrated on a 20 \times 0.1 mm nanotrap column (Thermo) equilibrated in 0.1 % TFA/water (10 μ l/min) and washed with 10 μ l of the same buffer. The samples were loaded and washed in TFA buffers, as the trap column in the presence of formic acid did not retain the tryptic peptide containing the Lys²⁹ linkage. Peptides were then separated on a 150 \times 0.075 mm PepMap C18, 3 μ m Easy-Spray column (Thermo) equilibrated with 2 % acetonitrile/0.1 % formic acid/water at 300 nl/min, employing a stepped gradient of buffer B (80 % acetonitrile/0.1 % formic acid/water) as follows: 0–14 min = 1 %–30 % B, 14–15 min = 30 %–80 % B, 15–20 min = 80 % B. LC–MS data was acquired in data-independent mode with one full scan (m/z = 350–1800) followed by eight product ion scans as described below. Parameters used: Easy-Spray column voltage was 1.9 kV; isolation width was set to 1 Da; normalized collision energy was 35, and the activation time was 10 ms. The ion current for the daughter ions was summed using Xcalibur software (Thermo) for each precursor mass analysed (Supplementary Table S2). The resultant summed intensities provide the y-axis values for Figure 1(B) and Supplementary Figure S2. This method was more specific than solely using the extracted ion current for the precursor mass for each Ub chain peptide.

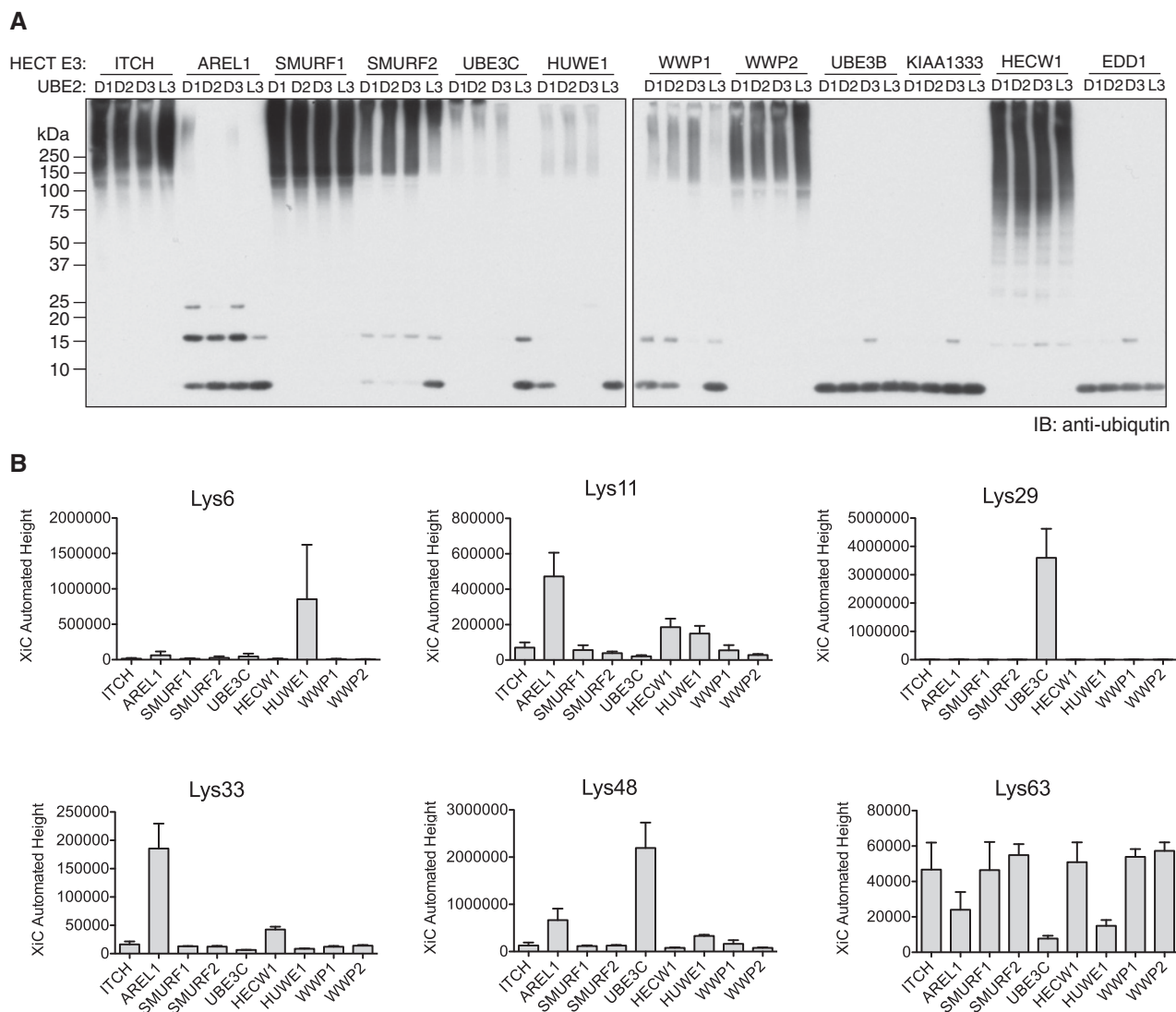


Figure 1 Screening of HECT E3 ligases to identify enzymes that assemble Lys³³-linked polyUb

(A) Ubiquitylation assays of HECT E3 ligases in the presence of UBE1, Ub and different E2 enzymes: UBE2D1, UBE2D2, UBE2D3 and UBE2L3. (B) Ubiquitylated products generated by HECT E3 ligases with the optimal E2 were analysed by pRM LC-MS/MS for the abundance of Ub linkages as described in 'Materials and Methods' (Supplementary Figure S1B). Each Ub linkage assembled by the HECT E3 ligases was plotted as a bar graph where the y-axes are summed ion current values for the relevant daughter ions of each precursor mass analysed (Supplementary Table S2). No signal was observed for Met¹ and Lys²⁷ linkages for any of the ligases tested.

Crystallization and structure determination

Purified Lys³³-linked diUb chains were crystallized at 9 mg/ml in mother liquor containing 200 mM lithium sulfate, 100 mM sodium acetate (pH 4.5) and 50 % PEG400. Further, diffraction quality of the crystal was improved using seeding technique in the presence of 200 mM potassium iodide and 20 % PEG3350 in addition to the mother liquor as mentioned above. Purified Lys³³-linked triUb chains were crystallized at 8 mg/ml in mother liquor containing 20 mM sodium/potassium phosphate, 100 mM Bis Tris propane (pH 7.5) and 20 % PEG3350. Single crystals obtained from Lys³³-linked diUb and triUb chains were cryo-protected in the mother liquor containing 20 % and 30 % ethylene glycol respectively. Diffraction data were collected at ESRF (European Synchrotron Radiation Facility) beam line ID29. All data were processed as in described in 'Supplementary Materials and Methods'. Co-ordinates and structure factors for the refined

Lys³³ diUb and triUb have been deposited in the Protein Data Bank (PDB, www.rcsb.org) under the accession code 4XYZ and 4Y1H respectively.

RESULTS AND DISCUSSION

Assembly of Lys³³-linked polyubiquitin

Lys¹¹, Lys⁴⁸ and Lys⁶³ chains can be assembled *in vitro* using E2 enzymes, whereas Lys⁶- and Met¹-linked polyUb can be assembled by HECT and RBR E3 ligases respectively [9,11,26,27]. Unlike RING E3 ligases, in which the linkage specificity is largely determined by the E2, polyUb assembly by HECT E3 ligases is independent of the inherent linkage preference of the E2 [7]. Therefore, we screened a panel of HECT E3s with the aim of identifying HECT E3 ligases capable of assembling

Lys³³ linkages. Either the full-length or the catalytic domains of 16 HECT E3 ligases were expressed as GST fusion proteins in *E. coli*. We obtained soluble expression for 12 of them, which were then purified to near homogeneity (Supplementary Figure S1A). We next determined the preferred E2 of a given HECT by comparing ubiquitylation products generated by the HECT in reactions performed with each of the following E2 enzymes: UBE2D1, UBE2D2, UBE2D3 or UBE2L3 (Figure 1A). Since HECT family ligases interact with UBE2L3 (UbcH7) and the UBE2D (UbcH5) subfamily of E2s, these E2 enzymes were selected for the screens [28,29]. With the exception of UBE3B, KIAA1333 and EDD1, all the tested HECT E3 ligases assembled polyUb chains (Figure 1A). Further, most of the HECTs work with UBE2D family members (Figure 1A). In this screen we found that AREL1 assembled shorter polyUb chains compared with the rest of HECT E3s. AREL1 might have slower kinetics as upon prolonged reaction time AREL1 also assembled longer chains (Supplementary Figure S1C). On the other hand, UBE3B, KIAA1333 and EDD1 failed to assemble polyUb chains, even after 6 h incubation (Figure 1A; Supplementary Figure S1C).

Next, we utilized MS to characterize the Ub linkages assembled by the different HECT E3 ligases (Supplementary Figure S1B). We analysed the products of the different HECT-mediated ubiquitylation reactions by parallel reaction monitoring (pRM) LC–MS/MS, a method that exclusively monitors the abundance of the daughter ions belonging to peptides derived from Ub linkages (Figure 1B) [30]. In accordance with previously published observations, we found that UBE3C assembles Lys²⁹ and Lys⁴⁸ linkages (Figure 1B) [31]. Further, most of the HECT E3s tested assembled Lys⁴⁸ and Lys⁶³ linkages similar to what had been observed previously [29,32]. Lys⁶ linkages are assembled mostly by HUWE1 and Lys¹¹ linkages are assembled by AREL1 and to some extent by HECW1 and HUWE1 (Figure 1B). Interestingly, our screen of HECT E3 ligases identified AREL1 as capable of assembling Lys³³ linkages (Figure 1B).

It is important to note that these *in vitro* screens assess polyUb linkages assembled by the HECT ligase in the absence of its bona fide substrate. In the presence of physiological substrates, these HECT E3s might assemble different linkages, preferring one linkage type over others. For example, Itch, which assembles Lys⁶³ linkages *in vitro* (Figure 1B), has been reported to modify its substrates with Lys²⁹ or Lys⁴⁸ linkages [33]. Additionally, Itch works co-operatively with RING E3 Cbl-b to ubiquitylate TCR- ζ with Lys³³ linkages. This suggests that additional factors may influence polyUb assembly by HECT E3 ligases.

Whereas AREL1 makes Lys³³ chains, it also assembles Lys¹¹ and Lys⁴⁸ linkages (Figures 1B and 2A). To obtain pure Lys³³-linked polyUb, the other linkages assembled by AREL1 have to be removed, for which linkage-selective DUBs are required. Cezanne mainly hydrolyses Lys¹¹ linkages, whereas OTUB1 only cleaves Lys⁴⁸ linkages [24]. We used a mutant version of Cezanne (Cezanne EK) that hydrolyses Lys⁶, Lys¹¹, Lys⁴⁸ and Lys⁶³ linkages (Supplementary Figure S2). When Cezanne EK and OTUB1 were included in the assembly reaction, the end product was enriched in free polyUb chains and almost 90% of the input Ub was converted into unanchored or free polyUb chains (Figure 2B). In order to confirm the linkage type of the resulting polyUb chains, we performed a linkage type analysis using Ub mutants containing lysine-to-arginine substitutions. In the presence of Cezanne EK and OTUB1, free polyUb chain formation was not impaired with K6R, K11R, K27R, K29R, K48R or K63R mutants (Figure 2C). In contrast, formation of polyUb chains was significantly reduced with the K33R mutant, suggesting that this method generates polyUb chains that are Lys³³ linked (Figure 2C). Moreover, when incubated with

Table 1 Data collection and refinement statistics

	Lys ³³ -linked diUb	Lys ³³ -linked triUb
Data collection		
Wavelength (Å)	0.999	0.976
Resolution range (Å)	33.83 to 1.65 (1.71 to 1.65)	31.42 to 1.40 (1.45 to 1.40)
Space group	P 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Unit cell a, b, c (Å)	29.48, 57.02, 33.98	28.94, 41.83, 47.60
α, β, γ (°)	90.00, 95.45, 90.00	90.00, 90.00, 90.00
Total reflections	58324 (5866)	74848 (7266)
Unique reflections	13474 (1348)	11652 (1127)
Multiplicity	4.3 (4.4)	6.4 (6.4)
Completeness (%)	99.40 (99.04)	98.06 (97.16)
I/ σ I	10.55 (4.20)	18.51 (12.82)
R_{merge}	0.1033 (0.2502)	0.0948 (0.1303)
CC1/2	0.99 (0.936)	0.99 (0.986)
Refinement		
Number of atoms		
Protein	1183	605
Ligand/ion	22	16
Water	99	36
$R_{\text{work}}/R_{\text{free}}$	0.165/0.214	0.158/0.194
RMSD		
Bond lengths (Å)	0.019	0.026
Bond angles (°)	1.98	2.28
Average B-factor (Å ²)	12.59	18.04

The highest resolution shell is shown in parentheses.

the DUB TRABID that specifically hydrolyses Lys²⁹ and Lys³³ linkages [23], the assembled polyUb chains were cleaved down to monoUb, confirming the presence of Lys³³ linkages (Figure 2D). Taken together, these results demonstrate that an Ub chain editing complex made up of the enzymes AREL1, UBE2D1, Cezanne EK and OTUB1 can be used to assemble Lys³³-linked polyUb chains.

We next scaled up the assembly reactions to make a large quantity of Lys³³-linked chains. Using cation exchange chromatography, we could separate Lys³³-linked chains of defined lengths containing 2–5 Ub moieties and the purity of Lys³³-linked diUb and triUb was confirmed by silver staining (Figures 2E and 2F). pRM LC–MS/MS analyses of purified diUb and triUb validated that the purified polyUb chains produced using this approach only contained Lys³³ linkages and other linkages were not detected (Supplementary Figure S2B). Taken together, these data reveal a robust and reproducible method for generating milligram quantities of Lys³³-linked polyUb.

Crystal structure of Lys³³ diubiquitin

The topology of polyUb of different linkage types and potentially the length of the polyUb chains determine specificity and outcome of polyUb recognition. We therefore wanted to structurally characterize Lys³³-linked polyUb chains. We obtained crystals of Lys³³-linked diUb at 9 mg/ml and the crystals diffracted to 1.65 Å (1 Å = 0.1 nm) resolution. The structure was solved by molecular replacement and refined to the statistics shown in Table 1. The asymmetric unit (ASU) contains one Lys³³-linked diUb (Figure 3A). The flexible isopeptide linkage formed between the C-terminus of the distal Ub and Lys³³ of the proximal Ub is not fully resolved in the electron density maps and no clear electron density is present for Gly⁷⁶.

Lys³³-linked diUb adopts a symmetric compact conformation in the crystal structure with extensive hydrophobic contacts between the proximal and distal moieties. Ile³⁶ patches of both proximal and distal Ub moieties, which comprise Ile³⁶, Leu⁷¹ and Leu⁷³, are

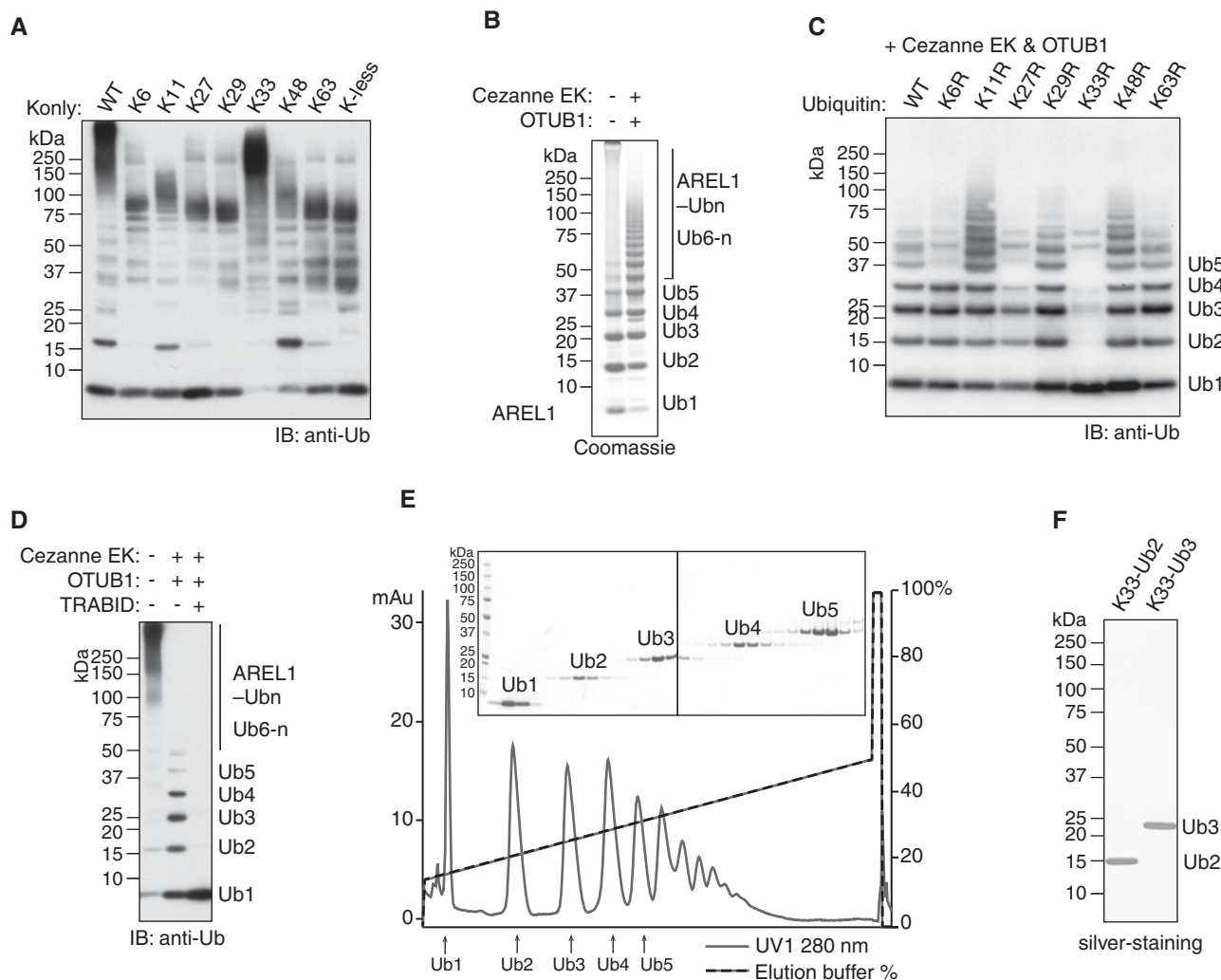


Figure 2 Assembly of Lys³³-linked polyUb

(A) Ubiquitylation assays of AREL1 in the presence of UBE1, UBE2D1 and wild-type Ub or Ub mutants that have only one or no lysine residue. (B) Large-scale assembly of polyUb chains by AREL1 in the presence of UBE1, UBE2D1 and Ub. The addition of DUBs, Cezanne EK and OTUB1, releases free polyUb chains. (C) Ubiquitylation assays of AREL1 in the presence of UBE1, UBE2D1 and wild-type Ub or lysine-to-arginine Ub mutants. DUBs, Cezanne EK and OTUB1, were added after 3 h of reaction. (D) Auto-ubiquitylation assays of AREL1 as in (A) with wild-type Ub. DUBs, Cezanne EK, OTUB1 and TRABID, were added after 3 h reaction as indicated. (E) Purification of Lys³³-linked chains of defined lengths by cation-exchange chromatography. (F) The Lys³³-linked diUb and triUb purified in (D) were visualized in silver-stained SDS gel.

present at the dimeric interface (Figure 3B). Further hydrophobic contacts in this symmetric interface involve Leu⁸, Ile¹³ and Leu⁶⁹ of both moieties (Figure 3B). Leu⁸ is part of a flexible loop in Ub that spans the β 1 and β 2 strands (β 1– β 2 loop) and exhibits different conformations in different Ub structures [34]. Depending on the conformation of this loop, Leu⁸ is part of either the Ile³⁶ patch or the orthogonal hydrophobic patch centred on Ile⁴⁴, consisting of residues Ile⁴⁴, Val⁷⁰ and His⁶⁸. In the observed Lys³³ diUb structure, this loop is oriented towards Ile⁴⁴ and is thus part of the Ile⁴⁴ patch (Figure 3C). In contrast, the β 1– β 2 loop conformation in the distal Ub of Lys⁶ diUb makes Leu⁸ part of the Ile³⁶ patch (Figure 3C) [8].

In the compact conformation of Lys⁶ diUb, the interface is made up of the extended Ile³⁶ patch from the distal Ub and the Ile⁴⁴ patch of the proximal Ub (Figure 3D). In Lys⁴⁸ diUb, the interface is made up of Ile⁴⁴ patches of both distal and proximal Ub (Figure 3D). The compact conformation observed for Lys³³ diUb is distinct from the compact conformations observed for Lys⁶ and Lys⁴⁸ diUb (Figure 3D) [8,9,12]. The Ile³⁶ patches of

both distal and proximal Ub in Lys³³-linked diUb are buried and make up the interface, whereas the Ile⁴⁴ patches form a larger hydrophobic surface and are solvent exposed. Molecular modelling approaches predict that Lys³³-linked diUb exists in an open conformation and cannot adopt a compact conformation due to steric occlusion [35]. However, our crystal structure reveals that Lys³³-linked diUb can adopt a closed conformation. Intriguingly, the closed conformation of Lys³³ diUb is very similar to that adopted by Lys¹¹-linked diUb (Figure 3D) [10]. Despite the similar conformations adopted by Lys¹¹ and Lys³³ linkages, DUBs can still distinguish between the two linkage types highlighting the remarkable selectivity present in the Ub system [24].

Crystal structure of Lys³³ triubiquitin

The presence of a symmetric interface raises the question of how chain extension can be achieved and what structure longer Lys³³ polyUb chains adopt. To address this question we purified

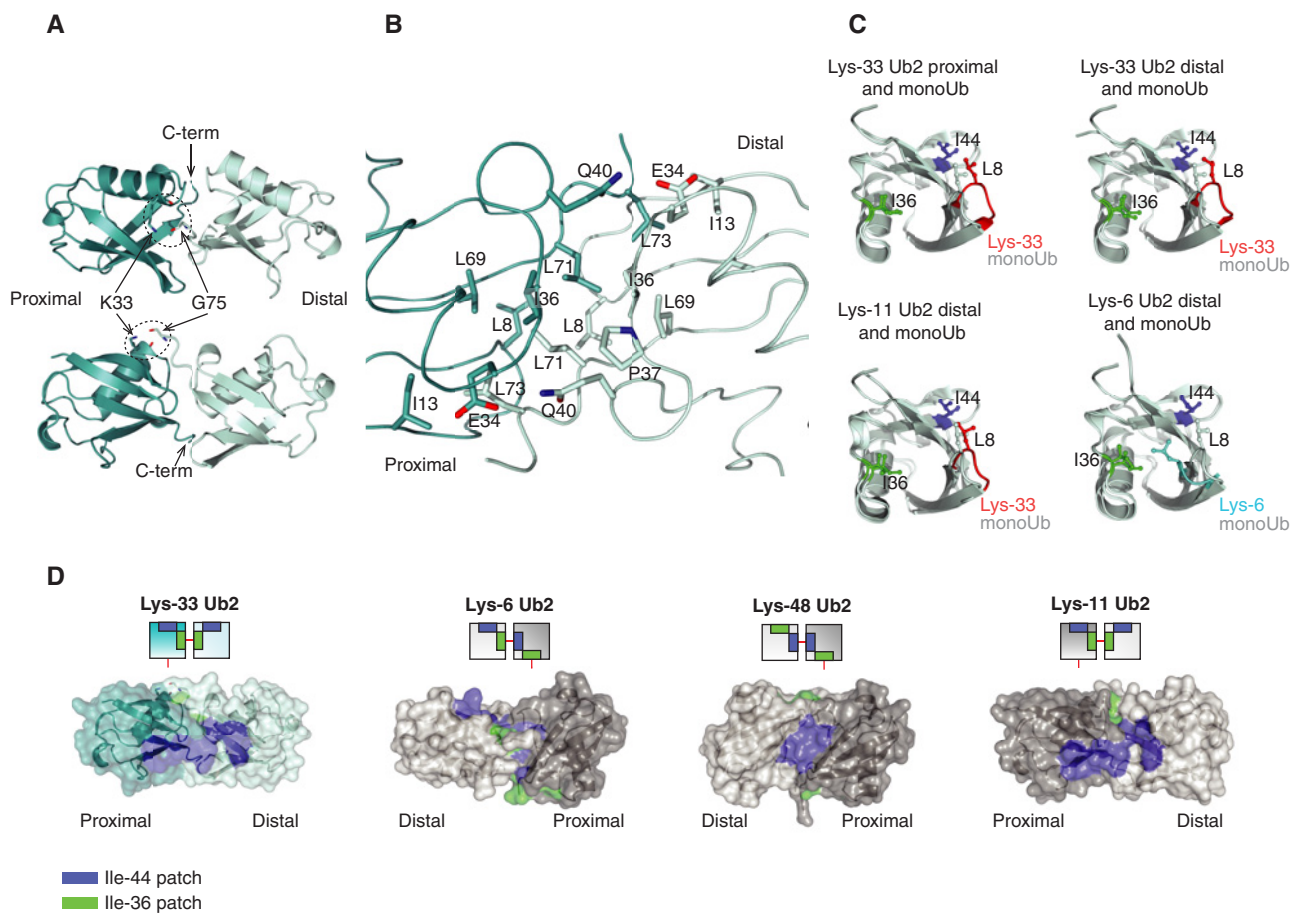


Figure 3 Crystal structure of Lys³³-linked diUb

(A) The crystal structure of Lys³³ diUb in two orientations. (B) Lys³³ diUb is shown in ribbon and the residues at the interface are shown in stick representation. (C) Leu⁸ residue of Lys³³-linked diUb contributes to Ile⁴⁴ patch. Proximal Ub of Lys³³ diUb and distal Ub of Lys³³ diUb, Lys¹¹ diUb (PDB 3NOB [10]) and Lys⁶ diUb (PDB 2XK5 [8]) were superposed with monoUb (PDB 1UBQ [41]) and coloured light cyan. The position of Leu⁸ (red), Ile³⁶ (green) and Ile⁴⁴ (blue) are indicated. As reference, the Leu⁸ of monoUb is not coloured differently. (D) A semi-transparent surface, coloured blue for Ile⁴⁴ patch (Ile⁴⁴, Leu⁸, His⁹⁶ and Val⁷⁰) and green for Ile³⁶ patch (Ile³⁶, Leu⁷¹ and Leu⁷³) of diUb linked via Lys³³, Lys⁶, Lys⁴⁸ and Lys¹¹ (PDB 2XK5, 3NOB, 1AAR [8,10,12]).

milligram quantities of Lys³³-linked triUb for crystallization studies. Lys³³ triUb crystallized in a different space group with unit cell dimensions different from that of Lys³³ diUb crystals. Diffraction data were obtained at 1.4 Å resolutions and the structure solved by molecular replacement and refined to the final statistics shown in Table 1. Although we crystallized triUb, the ASU only contains one Ub molecule (Figures 4A and 4B, chain B). In the crystal lattice, neighbouring Ub molecules complete the trimer where the C-terminus of a symmetry-related molecule (chain C) is close to Lys³³ of chain B; and the C-terminus of chain B is positioned close to Lys³³ residue of chain A (Figure 4A). Clear electron density is visible for the isopeptide linkage connecting the Ub moieties via Lys³³ (Supplementary Figure S3).

In contrast with the compact conformation adopted by Lys³³ diUb that involves extensive hydrophobic interactions at its interface, Lys³³-linked triUb adopts an open extended conformation. The three Ub molecules of the trimer are arranged in the same orientation forming a linear array, where there are no interactions between the individual Ub moieties apart from the isopeptide linkage (Figures 4A and 4B). When compared with the compact diUb, the proximal Ub of Lys³³-linked triUb is rotated by almost 65° suggesting lack of rotational constraints between individual Ub moieties (Figure 4C). Further, the hydrophobic patches are exposed to solvent, where symmetric arrangement

positions the Ile⁴⁴ hydrophobic patches on the same face of the trimer and the Ile³⁶ patches on another face (Figure 4D). This extended conformation of Lys³³ chains differs from the fully extended conformations observed for Lys⁶³ and Met¹ diUb [13]. In the crystal structures of Lys⁶³ and Met¹ chains, the hydrophobic patches alternate on opposite sides of the chain whereas they are located on the same face in Lys³³ chains (Figures 4D–4F).

Taken together, these results reveal two distinct conformations of Lys³³-linked Ub chains and the compact and extended conformations observed are distinct from those of Lys⁶, Lys⁴⁸, Met¹ and Lys⁶³ chains (Supplementary Figure S4). It is to be noted that the diUb structure was obtained from crystals grown at low pH (pH 4.5) whereas the triUb structure was from crystals grown at pH 7.5. This is in contrast with Lys⁴⁸ chains that adopt a compact conformation at physiological conditions and an open one at low pH (pH 4.5) [36,37]. The lack of intermolecular interactions in Lys³³-linked triUb suggests that the relative orientations of the Ub moieties may vary in solution, with our crystal structure representing a snapshot of this dynamic process. Further studies will be required to determine the preferred conformation of Lys³³ chains in solution.

The topology of polyUb chains together with the relative positioning and orientation of the hydrophobic patches are factors that determine linkage selectivity in polyUb binding.

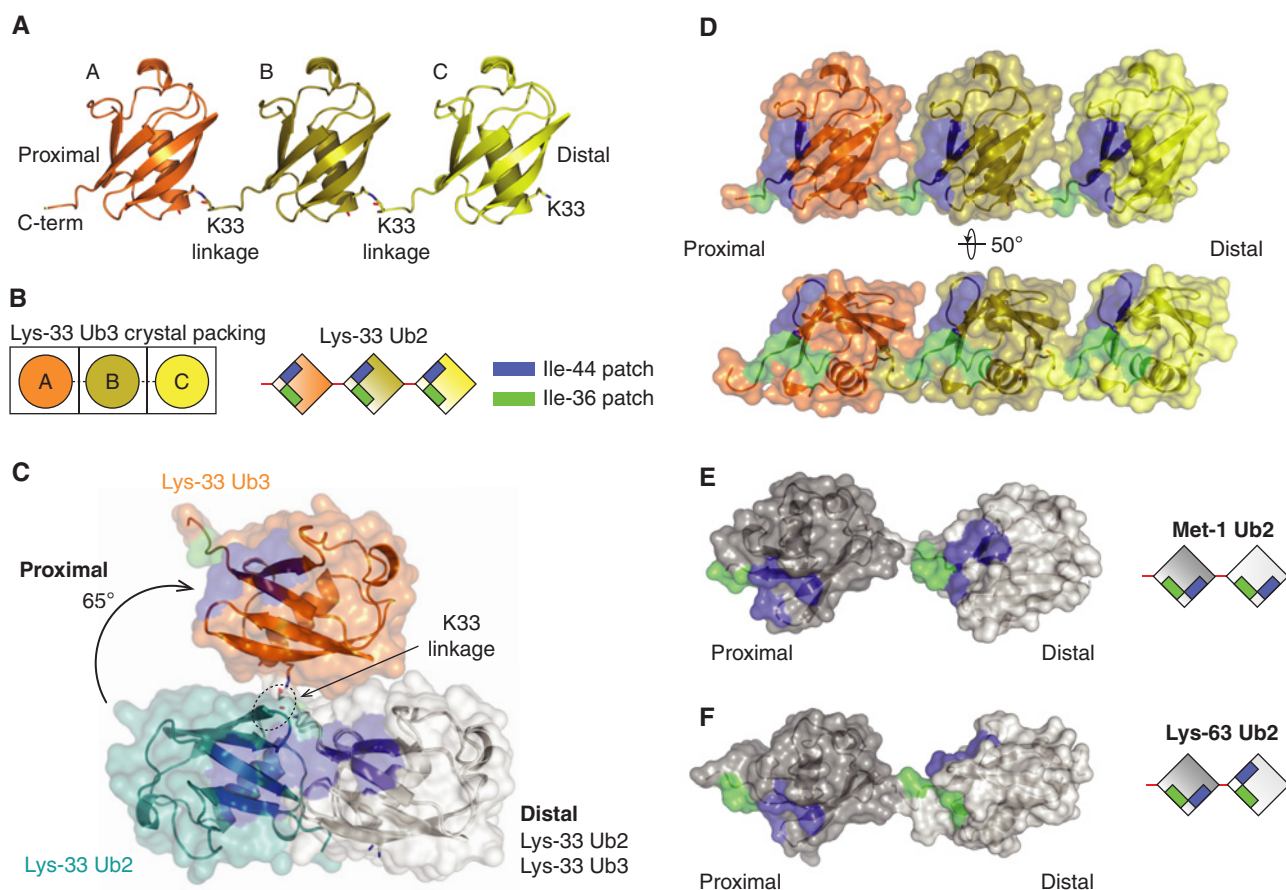


Figure 4 Crystal structure of Lys³³-linked triUb

(A) The crystal structure of Lys³³-linked triUb. (B) Representative diagram of how a Ub moiety in an ASU is arranged into triUb (left) and how Ile⁴⁴ and Ile³⁶ patches are located in Lys³³-linked triUb (right). (C) Superposition of the distal Ub moieties (white) of Lys³³-linked diUb and Lys³³-linked triUb, shown in cartoon and surface representations. Hydrophobic patches are coloured as in Figure 3D. Only two Ub moieties are shown for Lys³³-linked triUb. In comparison with the proximal Ub of Lys³³ diUb (teal), the proximal Ub of Lys³³ triUb (orange) moves approximately 65° from the more open conformation. (D–F) A semi-transparent surface of Lys³³ triUb (D), Met¹ diUb (E) and Lys⁶³ diUb (F) coloured as in Figure 3D (PDB 2W9N, 2JF5 [13]).

We have determined the structures of Lys³³-linked diUb and triUb that reveals compact and extended conformations with distinct characteristics. It will be important to analyse how UBDs and DUBs exploit the distinct features of Lys³³-linked polyUb to achieve linkage-selective recognition. The exposed hydrophobic patches, the unique structural features and the different conformations that can be adopted by Lys³³-linked polyUb are likely to be exploited by DUBs and UBDs. Further, shorter Lys³³ chains may have different conformations compared with longer chains and this introduces an additional layer of regulation where the length of the polyUb chain may determine which UBD binds and thereby determining the outcome of ubiquitylation.

AREL1 was recently identified as a novel anti-apoptotic E3 Ub ligase [38]. However, the Ub linkages assembled by AREL1 were not investigated. In our *in vitro* HECT E3 screen, we find that AREL1 mainly assembles Lys³³ and Lys¹¹ polyUb chains along with small amounts of Lys⁴⁸ and Lys⁶³ linkages. We speculate that AREL1 assembles mixed and branched polyUb chains containing different linkages. There is growing evidence suggesting specialized roles for mixed and branched chains [39,40]. Therefore, it will be important to address whether AREL1 assembles heterotypic chains in cells and what its cellular substrates are. Alternatively, AREL1 may be present in complex with DUBs that could promote ubiquitylation of substrates with

homotypic Lys³³ chains. Indeed studying the functional role of AREL1 may reveal insights into the biological roles of Lys³³-linked polyubiquitylation. Importantly, we provide the first description of an enzymatic system for the large-scale assembly of Lys³³-linked polyUb, which will pave the way for future studies.

AUTHOR CONTRIBUTIONS

Yosua Kristariyanto and Yogesh Kulathu designed, performed and analysed all experiments in the present manuscript. Soo-Youn Choi contributed to experiments. Syed Rehman performed some of the crystallographic analyses. Maria Ritorto, David Campbell and Nicholas Morrice performed MS analyses. Rachel Toth cloned all the DNA constructs used in the present study. Yogesh Kulathu and Yosua Kristariyanto wrote the manuscript with input from all authors.

ACKNOWLEDGEMENTS

We would like to thank A. Knebel, C. Johnson and R. A. Ewan for reagents; K. Airey and J. Stark for tissue culture support; and N. Helps for DNA sequencing. We thank P. Leznicki, M. Swamy and G. Sapkota for discussions and critical comments on the manuscript. Crystallographic data were collected at the European Synchrotron Radiation facility at beam line ID29. We thank the pharmaceutical companies supporting the Division of Signal Transduction Therapy (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck KGaA, Janssen Pharmaceutica and Pfizer).

FUNDING

This work was supported by the Medical Research Council U.K., Wellcome Trust [grant number 094090].

REFERENCES

- Haglund, K. and Dikic, I. (2005) Ubiquitylation and cell signaling. *EMBO J.* **24**, 3353–3359 [CrossRef PubMed](#)
- Pickart, C.M. and Eddins, M.J. (2004) Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta* **1695**, 55–72 [CrossRef PubMed](#)
- Deshaies, R.J. and Joazeiro, C.A.P. (2009) RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem.* **78**, 399–434 [CrossRef PubMed](#)
- Kee, Y. and Huijbregh, J.M. (2007) Regulation of catalytic activities of HECT ubiquitin ligases. *Biochem. Biophys. Res. Commun.* **354**, 329–333 [CrossRef PubMed](#)
- Spratt, D.E., Walden, H. and Shaw, G.S. (2014) RBR E3 ubiquitin ligases: new structures, new insights, new questions. *Biochem. J.* **458**, 421–437 [CrossRef PubMed](#)
- Ikeda, F. and Dikic, I. (2008) Atypical ubiquitin chains: new molecular signals. "Protein modifications: beyond the usual suspects" Review Series. *EMBO Rep.* **9**, 536–542 [CrossRef PubMed](#)
- Kulathu, Y. and Komander, D. (2012) Atypical ubiquitylation – the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nat. Rev. Mol. Cell Biol.* **13**, 508–523 [CrossRef PubMed](#)
- Virdee, S., Ye, Y., Nguyen, D.P., Komander, D. and Chin, J.W. (2010) Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase. *Nat. Chem. Biol.* **6**, 750–757 [CrossRef PubMed](#)
- Hospenthal, M.K., Freund, S.M. V. and Komander, D. (2013) Assembly, analysis and architecture of atypical ubiquitin chains. *Nat. Struct. Mol. Biol.* **20**, 555–565 [CrossRef PubMed](#)
- Matsumoto, M.L., Wickliffe, K.E., Dong, K.C., Yu, C., Bosanac, I., Bustos, D., Phu, L., Kirkpatrick, D.S., Hymowitz, S.G., Rape, M. et al. (2010) K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. *Mol. Cell* **39**, 477–484 [CrossRef PubMed](#)
- Bremm, A., Freund, S.M. V. and Komander, D. (2010) Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne. *Nat. Struct. Mol. Biol.* **17**, 939–947 [CrossRef PubMed](#)
- Cook, W.J., Jeffrey, L.C., Carson, M., Chen, Z. and Pickart, C.M. (1992) Structure of a diubiquitin conjugate and a model for interaction with ubiquitin conjugating enzyme (E2). *J. Biol. Chem.* **267**, 16467–16471 [PubMed](#)
- Komander, D., Reyes-Turcu, F., Licchesi, J.D.F., Odenwelder, P., Wilkinson, K.D. and Barford, D. (2009) Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep.* **10**, 466–473 [CrossRef PubMed](#)
- Hirano, T., Serve, O., Yagi-Utsumi, M., Takemoto, E., Hiromoto, T., Satoh, T., Mizushima, T. and Kato, K. (2011) Conformational dynamics of wild-type Lys-48-linked diubiquitin in solution. *J. Biol. Chem.* **286**, 37496–37502 [CrossRef PubMed](#)
- Rohaim, A., Kawasaki, M., Kato, R., Dikic, I. and Wakatsuki, S. (2012) Structure of a compact conformation of linear diubiquitin. *Acta Crystallogr. D Biol. Crystallogr.* **68**, 102–108 [CrossRef PubMed](#)
- Husnjak, K. and Dikic, I. (2012) Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu. Rev. Biochem.* **81**, 291–322 [CrossRef PubMed](#)
- Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K. and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243**, 1576–1583 [CrossRef PubMed](#)
- Xu, P., Duong, D.M., Seyfried, N.T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D. and Peng, J. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* **137**, 133–145 [CrossRef PubMed](#)
- Huang, H., Jeon, M.-S., Liao, L., Yang, C., Elly, C., Yates, J.R. and Liu, Y.-C. (2010) K33-linked polyubiquitination of T cell receptor-zeta regulates proteolysis-independent T cell signaling. *Immunity* **33**, 60–70 [CrossRef PubMed](#)
- Al-Hakim, A.K., Zagorska, A., Chapman, L., Deak, M., Pegg, M. and Alessi, D.R. (2008) Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains. *Biochem. J.* **411**, 249–260 [CrossRef PubMed](#)
- Yuan, W.-C., Lee, Y.-R., Lin, S.-Y., Chang, L.-Y., Tan, Y.P., Hung, C.-C., Kuo, J.-C., Liu, C.-H., Lin, M.-Y., Xu, M. et al. (2014) K33-linked polyubiquitination of coronin 7 by Cul3-KLHL20 ubiquitin E3 ligase regulates protein trafficking. *Mol. Cell* **54**, 586–600 [CrossRef PubMed](#)
- Reyes-Turcu, F.E., Ventii, K.H. and Wilkinson, K.D. (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu. Rev. Biochem.* **78**, 363–397 [CrossRef PubMed](#)
- Licchesi, J.D. F., Mieszczynek, J., Mevissen, T.E. T., Rutherford, T.J., Akutsu, M., Virdee, S., Oualid, F.E., Chin, J.W., Ova, H., Bienz, M. et al. (2011) An ankyrin-repeat ubiquitin-binding domain determines TRABID's specificity for atypical ubiquitin chains. *Nat. Struct. Mol. Biol.* **19**, 62–71 [CrossRef PubMed](#)
- Ritorto, M.S., Ewan, R., Perez-Oliva, A.B., Knebel, A., Buhlage, S.J., Wightman, M., Kelly, S.M., Wood, N.T., Virdee, S., Gray, N.S. et al. (2014) Screening of DUB activity and specificity by MALDI-TOF mass spectrometry. *Nat. Commun.* **5**, 4763 [CrossRef PubMed](#)
- Tran, H., Hamada, F., Schwarz-Romond, T. and Bienz, M. (2008) Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. *Genes Dev.* **22**, 528–542 [CrossRef PubMed](#)
- Kulathu, Y., Akutsu, M., Bremm, A., Hofmann, K. and Komander, D. (2009) Two-sided ubiquitin binding explains specificity of the TAB2 NZF domain. *Nat. Struct. Mol. Biol.* **16**, 1328–1330 [CrossRef PubMed](#)
- Stieglitz, B., Morris Davies, A.C., Koliopoulos, M.G., Christodoulou, E. and Rittinger, K. (2012) LUBAC synthesizes linear ubiquitin chains via a thioester intermediate. *EMBO Rep.* **13**, 840–846 [CrossRef PubMed](#)
- Kar, G., Keskin, O., Nussinov, R. and Gursoy, A. (2012) Human proteome-scale structural modeling of E2-E3 interactions exploiting interface motifs. *J. Proteome Res.* **11**, 1196–1207 [CrossRef PubMed](#)
- Sheng, Y., Hong, J.H., Doherty, R., Srikumar, T., Shloush, J., Avvakumov, G.V., Walker, J.R., Xue, S., Neculai, D., Wan, J.W. et al. (2012) A human ubiquitin conjugating enzyme (E2)-HECT E3 ligase structure-function screen. *Mol. Cell. Proteomics* **11**, 329–341 [CrossRef PubMed](#)
- Peterson, A.C., Russell, J.D., Bailey, D.J., Westphall, M.S. and Coon, J.J. (2012) Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Mol. Cell. Proteomics* **11**, 1475–1488 [CrossRef PubMed](#)
- You, J. and Pickart, C.M. (2001) A HECT domain E3 enzyme assembles novel polyubiquitin chains. *J. Biol. Chem.* **276**, 19871–19878 [CrossRef PubMed](#)
- Maspero, E., Mari, S., Valentini, E., Musacchio, A., Fish, A., Pasqualato, S. and Polo, S. (2011) Structure of the HECT:ubiquitin complex and its role in ubiquitin chain elongation. *EMBO Rep.* **12**, 342–349 [CrossRef PubMed](#)
- Chastagner, P., Israël, A. and Brou, C. (2006) Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. *EMBO Rep.* **7**, 1147–1153 [CrossRef PubMed](#)
- Lange, O.F., Lakomek, N.A., Fares, C., Schroder, G.F., Walter, K.F. A., Becker, S., Meiler, J., Grubmüller, H., Griesinger, C. and de Groot, B.L. (2008) Recognition dynamics up to microseconds revealed from an RDC-derived ubiquitin ensemble in solution. *Science* **320**, 1471–1475 [CrossRef PubMed](#)
- Fushman, D. and Walker, O. (2010) Exploring the linkage dependence of polyubiquitin conformations using molecular modeling. *J. Mol. Biol.* **395**, 803–814 [CrossRef PubMed](#)
- Lai, M.-Y., Zhang, D., LaRonde-LeBlanc, N. and Fushman, D. (2012) Structural and biochemical studies of the open state of Lys48-linked diubiquitin. *Biochim. Biophys. Acta* **1823**, 2046–2056 [CrossRef PubMed](#)
- Varadan, R., Walker, O., Pickart, C. and Fushman, D. (2002) Structural properties of polyubiquitin chains in solution. *J. Mol. Biol.* **324**, 637–647 [CrossRef PubMed](#)
- Kim, J.B., Kim, S.Y., Kim, B.M., Lee, H., Kim, I., Yun, J., Jo, Y., Oh, T., Jo, Y., Chae, H.D. et al. (2013) Identification of a Novel Anti-apoptotic E3 ubiquitin ligase that ubiquitinates antagonists of inhibitor of apoptosis proteins SMAC, HtrA2, and ARTS. *J. Biol. Chem.* **288**, 12014–12021 [CrossRef PubMed](#)
- Emmerich, C.H., Ordureau, A., Strickson, S., Arthur, J.S. C., Pedrioli, P.G. A., Komander, D. and Cohen, P. (2013) Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 15247–15252 [CrossRef PubMed](#)
- Meyer, H.-J. and Rape, M. (2014) Enhanced protein degradation by branched ubiquitin chains. *Cell* **157**, 910–921 [CrossRef PubMed](#)
- Vijay-Kumar, S., Bugg, C.E. and Cook, W.J. (1987) Structure of ubiquitin refined at 1.8 Å resolution. *J. Mol. Biol.* **194**, 531–544 [CrossRef PubMed](#)